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Original article

Transcriptome analysis reveals heat shock protein 70 (HSP70) induced by tembusu virus infection is associated with immune responses

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Abstract

The outbreak and prevalence of tembusu virus (TMUV) endanger the breeding industry of waterfowls. However, little is known about the molecular mechanism underlying TMUV infection. It was reported that heat shock protein 70 (HSP70) was a positive regulator of the infection of TMUV. In order to study the interactions between HSP70 and host immune response to TMUV infection, TMUV-infected cells with or without HSP70 inhibitor were harvested and subjected to deep sequencing to identify genes differentially expressed. We found 43 differentially expressed genes (DEGs) in HSP70 inhibitor-treated and mock-treated TMUV-infected DF-1 cells. Of these DEGs, 39 genes were down-regulated significantly. Gene Ontology analysis suggested that the DEGs were mainly involved in biological process, cellular component and molecular function. Kyoto Encyclopedia of Genes and Genomes analysis showed that the DEGs mainly related to the activation of innate immune response, including RIG-I-like receptor, toll-like receptor and NF-κB signaling pathway. Also, 12 down-regulated immune-related DEGs were selected for confirmation by reverse transcription quantitative real-time PCR verification, all these genes showed consistent expression between the result of reverse transcription quantitative real-time PCR and transcriptomic sequencing. These results revealed the important role of HSP70 in facilitating the innate immune response induced by TMUV infection. This is first to access the role of HSP70 in host response to TMUV infection, which provides a basis for further study of the pathogenesis of TMUV and contributes to the elucidation of TMUV-host interactions.

Keywords: heat shock protein 70, innate immune response, tembusu virus, transcriptome analysis

Introduction

Tembusu virus (TMUV) belongs to a family of viruses known as orthoflaviviruses, which includes Dengue virus (DENV), Zika virus (ZIKV), Japanese Encephalitis virus (JEV), West Nile virus (WNV) and Yellow Fever virus (YFV). TMUV mainly infects ducks and could cause severe symptoms. Almost all species of ducks infected by TMUV exhibit typical clinical symptoms including diarrhea, depression and paralysis. Moreover, TMUV infected egg-laying ducks exhibited a rapid decrease in egg production and hemorrhagic ovaritis (Hamel et al. 2021). Thus, huge economic losses have been caused to the major duck-producing provinces of China by sudden outbreak and fast spread of TMUV.

As obligate intracellular pathogens, viral replication rely on host factors. They may even exploit cellular functions to replicate their genome, assemble progeny virions and release (Puschnik et al. 2017). Many host components have been found to be involved in orthoflaviviruses cell infection. For example, orthoflaviviruses take advantage of cytoskeleton network, including molecular motors central and cytoskeletal-associated proteins, to facilitate their infection (Gerold et al. 2017). In addition, orthoflaviviruses rely on host molecular chaperones for folding and proteostasis (Taguwa et al. 2015). Heat shock protein 70 (HSP70), one of the most important stress-inducible chaperone, is reported to be required in multiple stages of the ZIKV, DENV, WNV and JEV infection (Pujhari et al. 2019). Our previous study demonstrated that HSP70 was highly expressed in TMUV-infected cells and its inhibition could lead to reduction in viral replication, assembly and release (Dai et al. 2022).

The host innate immunity is the first line of defense against pathogenic organisms. TMUV infection can stimulate the up-regulation of numerous innate immune related genes in ducks, resulting in the activation of immune signaling pathways, including the induction of the interferon, infammatory cytokines and antiviral proteins (Li et al. 2015). Although several studies have attempted to elucidate the duck immune responses to TMUV infection, the immune mechanisms and interaction between host factors and immune responses are still worth further exploration.

HSP70 is a highly conserved and ubiquitous intracellular protein, which exists at a nearly undetectable levels under normal conditions, but significantly induced by cellular stresses, such as pathogen invasion, heat stress and other stresses (Ye et al. 2013). HSP70 function virtually at all stages of the life of proteins from synthesis to degradation and plays a critical role in maintaining protein homeostasis (Rosenzweig et al. 2019). Besides, HSP70 has immuno-regulatory characteristics with both anti- and pro-inflammatory functions. Therefore, HSP70 has been designated as "chaperokine" (de Jong et al. 2009). HSP70 is involved in both the innate and adaptive immune responses. It can be recognized by immune cells and thus initiates signal transduction which in turn leads to the release of cytokines. It is reported that HSP70 activated the translocation of NF-κB to the nucleus and thus initiated transcription of genes resulting in the increased release of pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-12, IL-6 and TNF-α (Zininga et al. 2018). Conversely, the anti-inflammatory function of HSP70 is linked to activation of IL-10 production through TLR2-ERK signaling pathway (Borges et al. 2012). Nevertheless, to date, we know little about the interactions between HSP70 and host immune response in TMUV infection.

High-throughput RNA sequencing is an emerging technology with many advantages. It has become important approach for investigating cellular mechanisms and gene expression profiles involved in orthoflavivirus infections (Grifoni et al. 2018, Durham et al. 2019). In order to obtain the transcriptomic information and explore whether HSP70 cross-talks with the immune system, DF-1 cells infected by TMUV with or without HSP70 inhibitor were subjected to transcriptomic sequencing. Our data may clarify the interaction between HSP70 and the host immune response during TMUV infection and provide the candidate genes and key pathways targets for the prevention and control of TMUV.

Materials and Methods

Cells and viruses

Chicken embryo fibroblast DF-1 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum (FCS, HyClone). The TMUV JS804 strain (GenBank No. JF895923.2) was isolated from an affected goose with neurological clinical signs and propagated in DF-1 by our laboratory (Huang et al. 2013). The viral titer was determined based on the 50% tissue culture infective dose ($TCID_{50}$, Zhao et al. 2015).

Virus infection

DF-1 cells were seeded in 6-well plate and cultured for 24 h at 37°C, then pre-incubated with 5 μM HSP70 inhibitor MKT077 (Taguwa et al. 2015) or the corresponding concentration (5 μM) of DMSO for 2 h at 37°C. The treated cells were infected with TMUV at a multiplicity of infection (MOI) of 1. After 1 h

of viral infection, the inoculum was removed and new culture medium containing drug was added. Cells and culture supernatants were harvested at 24 h post infection. The viral nucleic acid was detected by reverse transcription quantitative real-time PCR and viral titres were determined by standard plaque assay as described previously (Dai et al. 2022).

RNA extraction, library construction and sequencing

Total cellular RNA was extracted using Trizol reagent (Beyotime) according to the manufacturer's protocol. RNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo) and checked using RNase free agarose gel electrophoresis. Then mRNA was captured from total RNA extraction by Oligo (dT) beads. The captured mRNA was cut into short fragments by mixing with fragmentation buffer and subsequently reverse transcripted into cDNA using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). After purification, double-stranded cDNA fragments were end repaired, adenosine residue added, and connected to Illumina sequencing adapters. The product was purified with 1.0× AMPure XP Beads. Next, ligated fragments with sequencing adapters were PCR amplified and size selected by agarose gel electrophoresis. The resulting library were sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Data analysis

Fastp (version 0.18.0) was used to get high quality clean data (Chen et al. 2018). Raw sequencing data were filtered by removing reads containing adapters, reads containing more than 50% of low quality (Q-value ≤ 20) bases and reads containing more than 10% of unknown nucleotides (N). The filtered data were aligned to the *Gallus gallus* (chicken) reference genome (Ensembl_release 106) using HISAT2.2.4 with "-rna-strandness RF" and other parameters set as a default. The mapped reads of each sample were assembled using StringTie v1.3.1 in a reference-based approach. The FPKM (fragment per kilobase of transcript per million mapped reads) value of each transcription region was calculated by RSEM software to quantify its expression abundance and variations (Li and Dewey 2011).

Identification of differentially expressed genes

Differential expression analysis was performed by DESeq2 (version 3.11) software between the group treated with HSP70 inhibitor and mock-treated group. The genes with absolute fold change higher than 2 and the parameter of false discovery rate (FDR) less than 0.05 were considered differentially expressed genes (DEGs). To recognize the main biological functions of DEGs, all DEGs were mapped to GO terms in the GO database, and hypergeometric test was used to find significantly enriched GO terms in DEGs comparing to the genome background. In addition, KEGG pathway analysis was implemented to identify significantly enriched signal transduction pathways or metabolic pathways in DEGs comparing with the whole genome background. Both GO and KEGG analysis, the calculated p-value were gone through FDR Correction, taking FDR equal or less than 0.05 as a threshold.

Reverse transcription quantitative real-time PCR verification

The DEGs revealed by RNA sequencing were validated by reverse transcription quantitative real-time PCR. Primers used in this study were listed in Table 1. The relative expression levels of target genes were calculated with comparative C_T method ($2^{-\Delta\Delta CT}$).

Statistical analysis

Each assay was replicated three times. Data were expressed as mean ± standard deviation (SD). Statistical differences between groups were analyzed using Student's t test. Statistical Package for the Social Sciences (SPSS, version 10.0) was used and value of p below 0.01 was regarded as having statistical significance.

Results

HSP70 inhibition results in reduction of TMUV viral RNA production and the number of virus particles

To confirm the role of HSP70 in TMUV infection, DF-1 cells were treated with HSP70 inhibitor MKT077. Then, infectious viral particles in the supernatants and the level of viral RNA were detected at 24 h post infection. Compared with mock-treated cells, a significant reduction of the viral RNA copies and titers was observed in MKT077 treated cells (Fig. 1). The results were consistent with our previous study that HSP70 exerted a positive effect on TMUV infection (Dai et al. 2022).

HSP70 inhibition alters the transcriptome profile during TMUV infection

Principal Component Analysis (PCA) was used to analyze the variance of within-group samples and

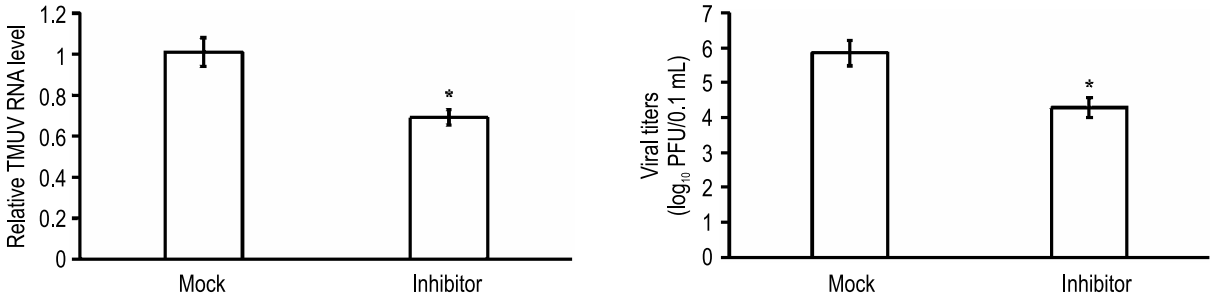

Fig. 1. Inhibition of heat shock protein 70 (HSP70) reduces tembusu virus (TMUV) viral RNA production and infectious titers. All experiments were performed in triplicate. Data are expressed as means ± standard deviation of the mean. The columns marked with the asterisks (*) show significant difference ($p<0.01$).

Table 1. Primers used for reverse transcription quantitative real-time PCR in this study.

Primer name	Sequence
IL-6F	5'-TGGTGATAAATCCCGATGAAG-3'
IL-6R	5'-GGCACTGAAACTCCTGGTCT-3'
IFN-αF	5'-ATGCCACCTTCTCTCACGAC-3'
IFN-αR	5'-AGGCGCTGTAATCGTTGTCT-3'
IFN-βF	5'-ACCAGGATGCCAACTTCT-3'
IFN-βR	5'-TCACTGGGTGTTGAGACG-3'
IFIH1F	5'-CTCTGCGAGAAACCCAACAT-3'
IFIH1R	5'-GCCCTCTGCTTCATCTTCAC-3'
OASLF	5'-ACATCCTCGCCATCATCGA-3'
OASLR	5'-GCGGACTGGTGATGCTGACT-3'
IFIT5F	5'-ACAAGGCACAAGAGGTTT-3'
IFIT5R	5'-TTTATCGGAGCAAGTCAG-3'
IL-1βF	5'-TGCTTCGTGCTGGAGTCAC-3'
IL-1βR	5'-GGCATCTGCCCAGTTCCA-3'
IKKαF	5'-TACTGCTTCCTGCCTGTGTG-3'
IKKαR	5'-GGAGGCCACAGTCCGTAAAA-3'
IKKβF	5'-GGGTCAGATCTGGCACACAA-3'
IKKβR	5'-TGGGAAAGGGAGGCCATAGA-3'
IRF7F	5'-GCCTGAAGAAGTGCAAGGTC-3'
IRF7R	5'-CTCTGTGCAAAACACCCTGA-3'
NLRP3F	5'-GCTCCTTGCGTGCTCTAAGACC-3'
NLRP3R	5'-TTGTGCTTCCAGATGCCGTCAG-3'
TLR3F	5'-TCAGTACATTTGTAACACCCCGCC-3'
TLR3R	5'-GGCGTCATAATCAAACACTCC-3'
POSTNF	5'-TGGAGCTGTCTATGAAACTTTGG-3'
POSTNR	5'-GAGTGTATTGGCCTTCTGGTCTT-3'
DF1-ACTINF	5'-CTGTGCCCATCTATGAAGGCTA-3'
DF1-ACTINR	5'-ATTTCTCTCTCGGCTGTGGTG-3'

correlations of between-group samples. The PCA plot demonstrated that transcriptome profiles of within--group samples were clustered together, and the expression profiles of transcripts changed between inhibitor-treated and mock-treated samples (Fig. 2A). In order to analyze transcriptomic response to HSP70 modulation during TMUV infection, we compared the transcriptome profiles of samples exposed to HSP70 inhibitor with those of mock-treated samples. As a result, 43 DEGs were identified. Of these DEGs, 39 genes (90.7%) were down-regulated, whereas 4 genes (9.3%) were up-regulated in expression, indicated that the overwhelming majority of genes were down-regulated under the treatment of HSP70 inhibitor (Fig. 2B-D).

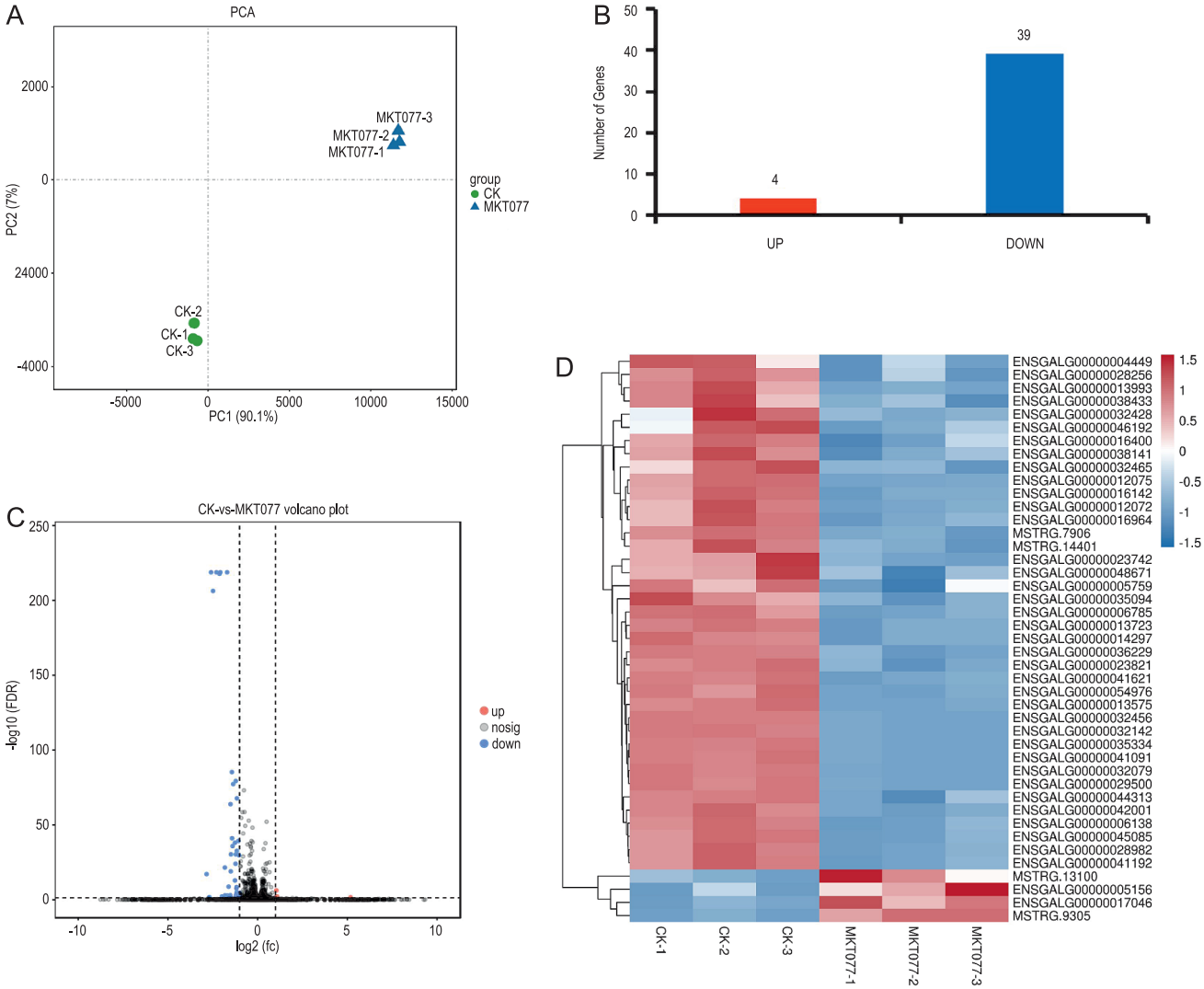

Fig. 2. Transcriptome analysis of gene expression in HSP70 inhibitor-treated and mock-treated TMUV-infected DF-1 cells. A – Principal Component Analysis (PCA) of the RNA-seq data. The blue triangles indicate samples from the HSP70 inhibitor-treated TMUV-infected group, and green dots indicate the mock-treated TMUV-infected cells. Each treatment group consists of three independent biological replicates. B – Bar graph showing the total number of DEGs between two treatment groups. C – Volcano plot of DEGs. D – Hierarchical clustering and heatmap of DEGs between two treatment groups. CK-1, CK-2, and CK-3 represent three independent biological replicates from HSP70 inhibitor-treated cells. MKT077-1, MKT077-2 and MKT077-3 represent three independent biological replicates from mock-treated cells.

GO enrichment analysis

GO enrichment analysis can filter the DEGs that correspond to the biological functions. Functional analysis revealed that all the DEGs could be grouped into three categories including biological process, cellular component and molecular function. There were 17 clusters in biological process, 11 clusters in cellular component and 6 clusters in molecular function (Fig. 3A). In the biological process category, most of the down-regulated DEGs were enriched in clusters including cellular process, metabolic process, response to stimulus, immune system process and positive regulation of biological process. Among clusters in cellular component, majority of down-regulated DEGs were involved in cell, cell part, organelle, organelle part and membrane part. As for molecular function, the down-regulated DEGs were mainly enriched in clusters including catalytic activity, binding, transporter activity and molecular function regulator. Further analysis indicated that down-regulated DEGs mainly enriched in GO terms including response to virus, immune effector process, regulation of innate immune response, defense response to virus, positive regulation of innate immune response and activation of innate immune response (Fig. 3B). Therefore, it is possible that HSP70 inhibition during TMUV infection modified immune response process through altering the expression level of key genes in three categories of DEGs.

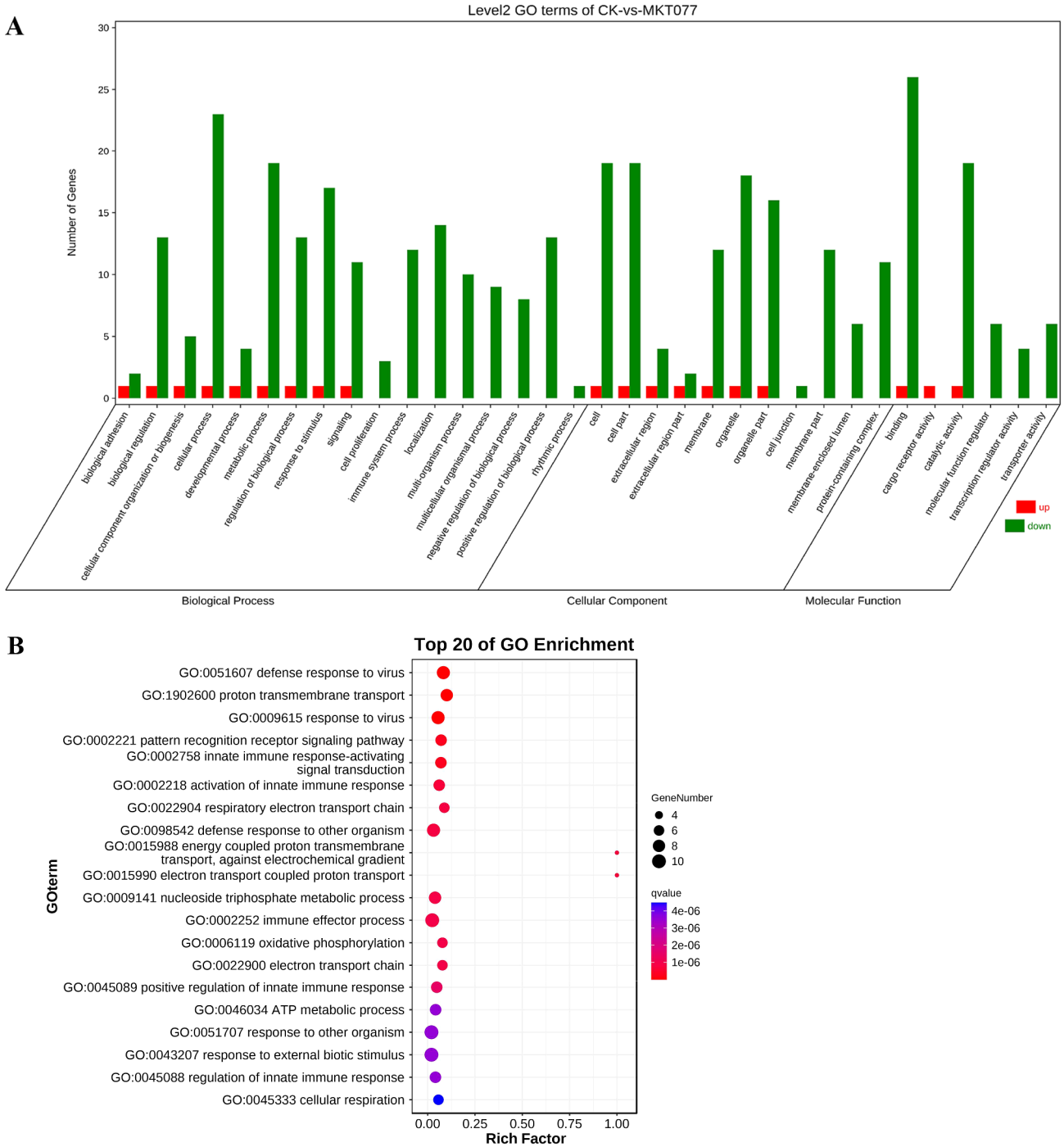

Fig. 3. Gene Ontology (GO) analysis of differentially expressed genes (DEGs). A, Histogram of GO classification of the DEGs. The GO distributions of the DEGs were classified into three major categories: "biological process", "cellular component" and "molecular function". B, Top 20 enriched GO biological process terms.

KEGG pathway enrichment analysis

Pathway-based analysis helps to further understand genes biological functions. Thus, we performed KEGG enrichment analysis to elucidate DEGs function. In the KEGG analysis, oxidative phosphorylation, Parkinson disease, RIG-I-like receptor signaling pathway, influenza A and other pathways were enriched. Hence, the results demonstrated that genes in these pathways may be involved in response to HSP70 inhibition during TMUV infection. The immune-related genes involved in the pathways were listed in Table 2.

Verification of DEGs by reverse transcription quantitative real-time PCR

Reverse transcription quantitative real-time PCR was used to verify the expression findings obtained

Table 2. Immune-related genes in various immune pathway were enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

Pathway (ID)	Gene	Gene ID	Style
RIG-I-like receptor signaling pathway (ko04622)	*IFNW1*	ENSGALG00000005759	Down
	IRF7	ENSGALG00000014297	
	DHX58	ENSGALG00000023821	
	IFIH1	ENSGALG00000041192	
	TRIM25	ENSGALG00000048671	
NF-kappa B signaling pathway (ko04064)	*CCL19*	ENSGALG00000028256	Down
	CCL26	ENSGALG00000046192	
	TRIM25	ENSGALG00000048671	
TNF signaling pathway (ko04668)	*IFNW1*	ENSGALG00000005759	Down
	IRF1	ENSGALG00000006785	
Toll-like receptor signaling pathway (ko04620)	*IFNW1*	ENSGALG00000005759	Down
	IRF7	ENSGALG00000014297	
JAK-STAT signaling pathway (ko04630)	*IFNW1*	ENSGALG00000005759	Down
	IL11	ENSGALG00000054976	
NOD-like receptor signaling pathway (ko04621)	*IFNW1*	ENSGALG00000005759	Down
	IRF7	ENSGALG00000014297	

from transcriptomic sequencing. We selected twelve genes that were involved in immune response to viruses and one up-regulated DEG for validation. The results obtained from reverse transcription quantitative real--time PCR were consistent with RNA sequencing data. As shown in Fig. 4, HSP70 inhibitor treatment remarkably decreased the expression of the immune-related genes at 24 h post infection, substantiating the reliability of RNA sequencing results.

Discussion

Since 2010, TMUV has become an important pathogen that endangers waterfowl, and continues to cause huge economic losses in the poultry industry in China and Southeast Asia every year. Up to now, great progress has been made in the interaction between TMUV and host innate immune response. However, little is known about the role of HSP70 in TMUV-induced host immune response. Our results revealed association between HSP70 and host immune response after TMUV infection.

Toll-like receptors 3 (TLR3) recognizes viral double-stranded RNA (dsRNA) and dsRNA produced during the course of replication of single-stranded RNA such as orthoflaviviruses. Melanoma differentiation associated protein 5 (MDA5), encoded by the IFIH1 gene, is a RIG-I-like receptor dsRNA helicase enzyme. It binds long dsRNA and is also required for the recognition of RNA viruses. TLR3 and MDA5 are implicated in triggering immune responses by producing inflammatory cytokines and type I IFN (Kawai and Akira 2009). Previous investigations have proved that TMUV infection can effectively up-regulate the expression levels of TLR3 and MDA5 mRNA and trigger host innate immune response through TLR3 and MDA5--dependent signaling pathways (Chen et al. 2016). Here, we found that the inhibition of HSP70 significantly reduced the RNA level of MDA5 and TLR3, suggesting that HSP70 performed a positive role in the activation of host innate immune response.

As IRF3 is absent in poultry, IRF7 may be an important regulator of IFN-α/β induction in virus-infected avian-origin cells. Previous studies showed that IRF7 regulated host anti-TMUV immune response, possibly depending on activation of IFN-α/β and associated signaling pathway (Chen et al. 2019). IFN-α/β can activate the transcription and translation of IFN-induced protein with the tetratricopeptide repeats 5 (IFIT5, Rauch et al. 2013). Wu et al. (2020) found that TMUV could significantly induce the transcription of IFIT5. Our data revealed that expressions of IFN-α/β, IRF7 and IFIT5 were significantly decreased in HSP70 inhibitor-treated cells as compared to mock-treated cells. 2'-5'-Oligoadenylate synthetase-like protein (OASL) is one of the most predominant IFN-stimulated genes (ISGs) and functions in cellular innate antiviral response (Chen et al. 2017). Our results showed that down-regulation of IFN-α/β expression by HSP70 inhibition lead to a reduction of OASL expression. Based on these data, we assume that HSP70 possibly regulate the signaling pathway involved in IFN system. Although OASL and IFN-α/β were down-regulated,

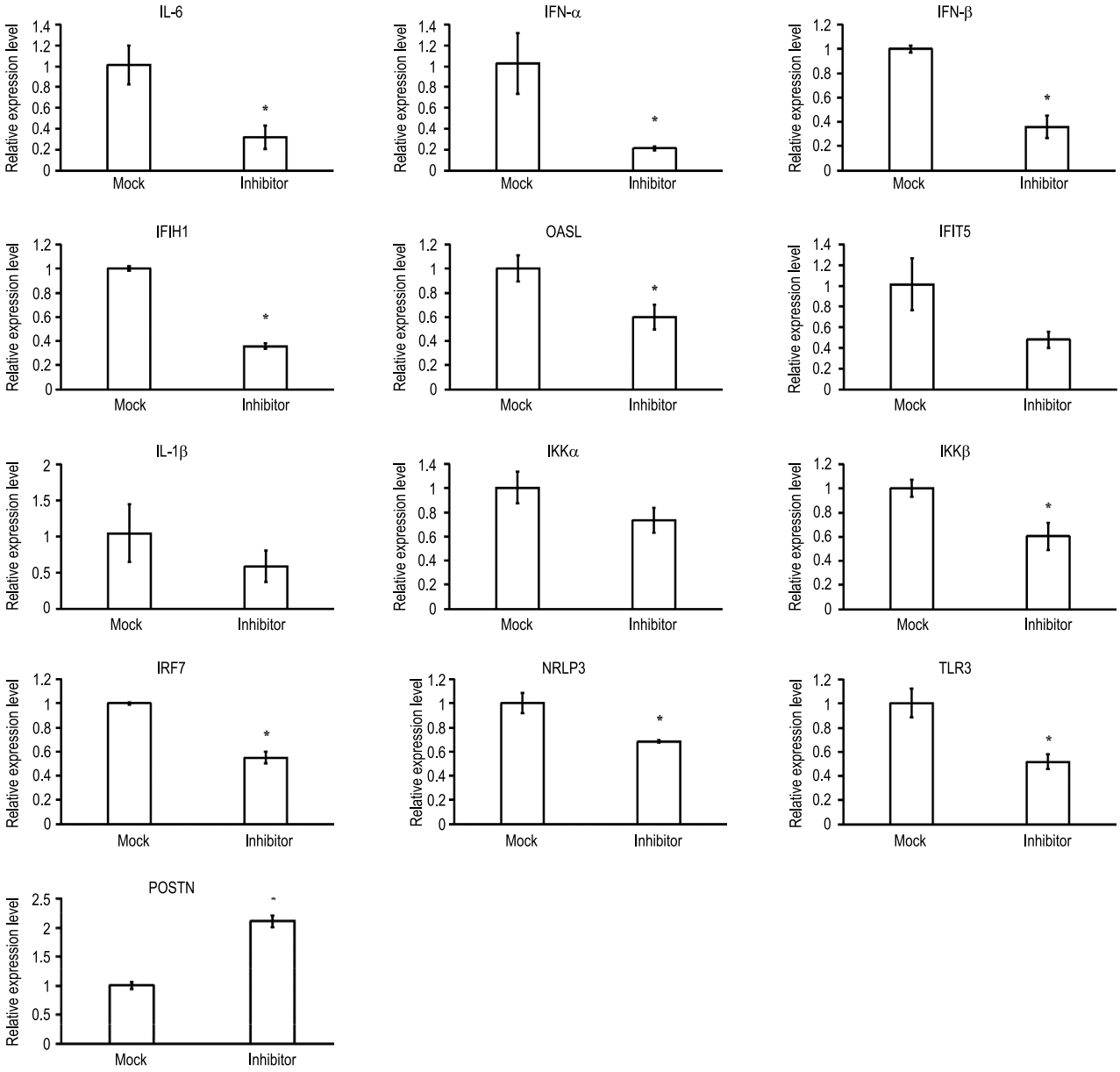

Fig. 4. Verification of the transcriptomic sequencing data by reverse transcription quantitative real-time PCR. Twelve DEGs involved in immune-related pathways were chosen for verification by quantitative real-time PCR. The relative expression level was quantified using the comparative CT method ($2^{-\Delta\Delta CT}$). β-actin was chosen as an internal reference gene and the mock-treated TMUV-infected cells were employed as a reference for each comparison (relative expression = 1). Data are expressed as the means ± standard deviation of the mean (n=3). The asterisk (*) represents a statistically significant difference ($p<0.01$).

the viral RNA copies and titers was significantly reduced in HSP70 inhibitor-treated cells as compared to mock-treated cells. As shown in our earlier study, HSP70 is a positive regulator of TMUV replication and required at multiple steps of the TMUV life cycle including viral replication, assembly and release (Dai et al. 2022). Therefore, the reason for this may be that down-regulated OASL and IFN-α/β were not adequate to counteract the effects of HSP70 inhibition on TMUV infection. However, our findings are very superficial, and the regulatory mechanism needs to be further investigated.

HSP70 acts as both a molecular chaperone and a cytokine (Asea et al. 2000). It is thought to facilitate pro-inflammatory response by inducing cytokines. HSP70 plays a role in NF-κB and its entry into the nucleus where it enhances transcription of genes resulting in the up-regulated expression of IFN, IL-6 and IL-1β (Zininga et al. 2018). Therefore, in this study, the inhibition of HSP70 leads to down-regulation of a series of immune-related genes but not due to the decreasing of the viral titer.

The robust production of interleukin 6 (IL-6) and IL-1β was observed during TMUV infection in vivo and in vitro. Massive IL-6 and IL-1β expression may cause the cytokine storm and contribute to more severe

pathological lesions (Li et al. 2015, Wang et al. 2018). In this study, the expression levels of IL-6 and IL-1β decreased by approximately 70% and 50%, respectively, after treatment with HSP70 inhibitor. Likewise, HSP70 inhibition lead to reduction of NLRP3 expression when compared to the control cells. Based on these data, we speculated that HSP70 may be involved in inflammatory response induced by TMUV infection.

Nuclear factor-κB (NF-κB) is a critical regulator that controls numerous genes expression involved in immune and inflammatory response (Liu et al. 2017). Although NF-κB was activated by distinct nodes, the majority of the signaling cascades converge on the activation of the IKK complex (Scheidereit 2006). In this study, the expression of IKKα and IKKβ was reduced in HSP70 inhibitor-treated cells as compared to the mock-treated cells, implying the reduction of NF-κB activation. The role of HSP70 in NF-κB-mediated inflammatory response is quite broad. Salminen et al. (2008) showed that HSP70 inhibited the activation of IKK complex by causing the dissociation of IKKα and IKKβ. On the contrary, Kumada et al. (2019) proved that HSP70 stabilized the IKKβ/IκBα/NF-κB p65 complex and facilitated the phosphorylation of NF-κB p65. However, those studies enumerated above were carried out in mammals, to the best of our knowledge, there is no report on the function of HSP70 in the activation of NF-κB in avian. Our findings suggested that HSP70 probably promoted NF-κB activation through regulating the expression of IKKα and IKKβ, but further studies are necessary to elucidate the exact mechanism of HSP70's role in NF-κB activation and innate immune response.

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